EMSA

Preparing of cell extracts

Cells (e.g. 293 cells) of one 6-well (10 cm^2, app. 10^6 cells): add 100 µl/well:

1x EMSA lysis buffer (stock sol.: 5x):

- 10 mM Tris/HCl pH 7.5
- 1 mM EDTA
- 5 mM MgCl
- 50 mM KCl
- 1 mM DTT
- 1x protease inhib. (Complete)

- Lysis of cells by 4 freeze/thaw cycles (-80°C/37°C incubator): on the plates,
- check breakage by microscopy
- suspend with pipette and transfer to Eppendorf tubes > 1 additional freeze/thaw cycle
- centrifugation: 14 000 rpm, 4°C, 15 min > take supernatant: measure vol.: approx.75 µl (> can be frozen at -70°C)
- add glycerol to 10% final conc., add KCl to 150 mM (4.2 µl 1 M to 75 µl sample)

Determine protein concentration of the extracts with Bradford reagent:

standards: BSA: 0, 1, 2, 3, 4 µg (µl) in 96well plates
extracts: 1 µl each (or diluted);
+ Biorad Bradford reagens (1:5, 200 µl) > measure OD595 in a microtiter plate reader
expected concentration of extracts: 2 – 3 µg/µl

Annealing of oligos

- equimolar amount of sense and antisense oligo: 400 pmol each (approx. 5 µg): 4 µl
- 20 µl 10x Buffer B (Roche)
- A.dest. ad 200 µl (172 µl)
- heat to 95°C (5 min)
- switch off the thermoblock and let cool down to RT

concentration: 400 pmol/200 µl = 2 pmol/µl

Labeling of annealed oligo with ^{32}P-alpha-dATP with TdT

(Fermentas #EP0161, Terminal deoxynucleotide Transferase)

- 10 µl 5x reaction buffer
- 2.5 µl annealed oligo (10 pmol of 3' termini = 5 pmol ds-oligo)

- 5 µl ^{32}P-alpha-dATP (10 µCi/µl > 50 µCi)
- 2 µl TdT (40 u)
- 30.5 µl A.dest. nuclease free

incubate at 37°C for 15 min
(Stop the reaction by heating to 70°C for 10 min).

Incubation of extracts with oligos and native PAGE

(Electrophoresis on PhastSystem, GE Healthcare, formerly Pharmacia-Amersham: see instruction manual of the manufacturer)

- 2 µl extract,
- 1 µl 1x EMSA lysis buffer (or competitor > 20x molar excess)
- 0.5µl ^{32}P-Oligo

> incubated 15 min at RT

+ 1 µl 1x EMSA lysis buffer (+ small amount bromphenolblue)

- pipetted into 4 µl sample combs of the PhastSystem
- run samples on 12.5% homogenous Phastgels using native buffer strips

Sample Appl. down at 4.2.	0Vh			
Sample Appl. up at 4.2		2Vh		
Step 4.1. 400 V	10.0 mA	2.5W	15°C	10 Vh
Step 4.2. 400 V	1.0 mA	2.5W	15°C	2 Vh
Step 4.3. 400 V	10.0 mA	2.5W	15°C	140 Vh